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PRINCIPAL INVESTIGATOR: Katherine Kennedy, Ph.D.

CONTRACTING ORGANIZATION: The George Washington University
Washington, DC 20037

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Katherine Kennedy, Ph.D.

Email: phmkak@gwumc.edu

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14. ABSTRACT
Physiological stress conditions associated with solid tumors play a role in chemotherapeutic resistance. Treatment with hypoxia or chemical stress agents causes EMT6 mouse mammary tumor cells to develop resistance to teniposide and etoposide, a topoisomerase II inhibitor. We have shown that prostaglandin A1 can fully reverse stress-induced resistance to teniposide or etoposide and the PGA1 can reverse this resistance when given either prior to or after the stress. PGA1 could also block activation of the transcription factor, NF-kB, as measured by gel shift assays or a luciferase reporter gene. To test whether NF-kB was directly involved in stress-induced resistance, an inducible promoter plasmid system containing a mutant IkB gene (which was non-phosphorylatable) was introduced into EMT6 cells as a dominant negative mutant. Expression of the dominant negative mutant prevented the stress activation of NF-kB and reverted the resistant phenotype to a drug sensitive phenotype. These results imply that NF-kB directly mediates both chemical and physiological stress-induced drug resistance in cancer cells and suggest that agents like PGA1 which prevent NF-kB activation may improve the efficacy of topoisomerase II inhibitors.

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Year 2 Report

Year 1 Report

Overall Summary of Key Accomplishments for DAMD17-99-1-9186

- Determined that prostaglandin A_1 inhibited both brefeldin A and hypoxia induced activation of NFκB
- Determined the prostaglandin A_1 reversed both hypoxia and brefeldin A induced toxicity to etoposide and teniposide. Prostaglandin A_2 can cause partial or complete reversal when given prior to or up to 7 hours after stress.
- Prostaglandin A_1 does not alter topoisomerase II activity directly.
- The stress agent, brefeldin A does not directly effect topoisomerase II activity directly nor does it alter drug uptake in cells.
- Constructed the pIND-IκBαM plasmid which contained the nonphosphorylatable form of IκB.
- Transfected pIND-IκBαM and pVgRXR plasmids into EMT6 cells and isolated hygromycin resistant clones.
- Showed that ponasterone A, the ecdysome receptor ligand, does not alter NFκB activation and does not effect long term survival of EMT6 cells.
- Showed that ponasterone A induction of IκBαM cells resulted in the expression of IκBαM protein and that ponasterone A induction of IκBαM cells resulted in the inhibition of stress-induced activation of NF-κB.
- Showed that induction of IκBαM cells resulted the diminution of measurable NFκB complexes in gel shift assays with brefeldin A, hypoxia or okadaic acid as the stress agent.
- Induction of IκBαM in nonstressed cells did not alter cellular sensitivity ot etoposide per se.
- Induction of IκBαM in stressed cells reversed stress induced resistance to teniposide.
- Demonstrated that the dose dependent induction of p65,p50, and IkBM tranfected cells by ponasterone resulted in the dose dependent expression of the proteins and effects on drug resistance to etoposide.
- Showed that dose dependent induction of p65 and p50 cells by ponaterone led to increased NFkB activity as measured by elevation of luciferase.
- Used expression arrays to begin to dissect the common downstream pathways for NFkB mediated drug resistance.
- Demonstrated that stress induced activation of NFkB controls TGF-beta expression in these cells and leads to a signal transduction pathway that contributes to drug resistance.
- Identified 40-80 genes thatplay a possible role in mediating resistance and resistance reversal.
- Demonstrated that human breast tumor cell lines exhibit similar patterns of resistance to topoisomerase II inhibitors after physiologic or chemical induced stress.
- Demonstrated that human breast tumor cell lines respond to stress in a qualitatively similar fashion with activation of NFkB.

- Demonstrated that PGA1 could sensitize human breast cell lines to topoisomerase II inhibitors.
- Demonstrated that human tumor cell lines such as PC3 and H1299 cells have patterns of response to stress similar to breast cell lines.
- Demonstrated that p53 status does not alter the response of cells to stress induced drug resistance but does shift the dose response curve.
- Demonstrate that p53 status does not alter sensitization to drug by NFkB inhibitors such as PGA1.
- Demonstrated that repeated exposure to stress (chemical or physiologic) results in an exaggerated more robust resistance to drug and that the response is more long lasting in that it does not disappear until after 8 passages.
- Demonstrated that hypoxia appears to blunt the development of drug induced acquired resistance.
- Demonstrated that repeated stress exposure cannot be explained by changes in NFkB activation, induction of HIF-1, or induction of VEGF since these are activated or induced to the same extent as control cells when stressed.

Year 3 and Extension

Introduction

The success of breast cancer treatment is often limited by the development of drug resistance. A unique form of drug resistance has been described that is induced by the physiological conditions associated with solid tumors. EMT6 mouse mammary tumor cells treated with hypoxia or the chemical stress agent brefeldin A (BFA) induce resistance to agents that inhibit topoisomerase II (Lin *et al*., 1998). Our data have shown that hypoxia- and BFA-induced resistance to topoisomerase II inhibitors is not mediated by alterations in p-glycoprotein, drug concentration, or topoisomerase II activity. Our recent data suggest that chemically-induced resistance is mediated by the nuclear transcription factor NF-κB (Lin *et al*., 1998). The overall scope of the work is to determine if NF-κB is the mediator of physiologic induced resistance and whether agents which alter NF-κB activation can alter the effectiveness of topoisomerase II type drugs.

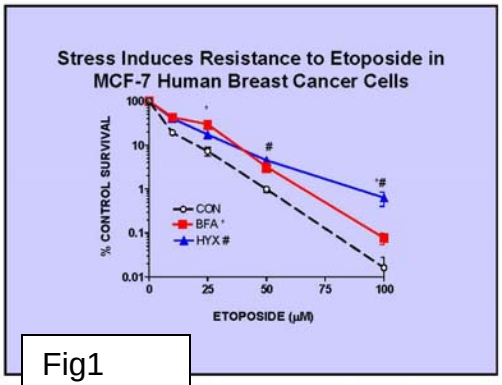
Body

Task 4 was to transfect into EMT6 cells the non-phosphorylatable mutant of IκB to produce a dominant negative mutant EMT6 cell line. **Task 5** was to evaluate the responses of the cell lines to stress. Our experiments with the that the ecdysone inducible system showed that ponasterone induced expression of IκBαM in the cells; that expression of the IκBαM protein prevented NF-κB activation by brefeldin A (BFA), hypoxia (H), or okadaic acid(OA); that the presence of the IκBαM vector or the expression of IκBαM protein in the cells did not alter the cytotoxic effects of etoposide in EMT6 cells in the *absence* of stress; that hypoxia and brefeldin A both induced resistance in EMT6 cells containing either the vector only construct or the uninduced IκBαM construct; and that induction of IκBαM expression with ponasterone A prevented the development of stress-induced resistance (induced by hypoxia or brefeldin A) to etoposide. In fact, ponasterone A induced cells were not statistically different from non-transfected EMT6 cells in their susceptibility to etoposide and doxorubicin.

We continued the work in EMT6 cells by extending it to human breast and other tumor cells lines. These data were obtained from MCF7 estrogen receptor positive cells, MDA-MB 231 estrogen receptor negative cells, PC3 human prostate cancer cells, H1299 human lung cancer cell lines which were either p53 positive or p53 negative.

Both MCF7 and MDA-MB 231 human breast tumor cells showed typical patterns of drug resistance when pretreated with stress (Figures 1-3). Both hypoxia and chemical stress caused activation of NF-kB (Figures 4, 5). Pretreatment with PGA1, which inhibits NF-kB activation, sensitized breast cells to etoposide (Figures 6 and 7). These responses demonstrated that human breast tumor cell

lines behave similarly to EMT6 cells in their response to physiologic and pharmacologic induced stress. Figures 8-10 demonstrated that these same responses extend to the human prostate cancer cell line PC3. In evaluating, these cell lines, they were detemined by comparison to have relatively high constitutive levels of NFkB in agreement with suggestions by others.



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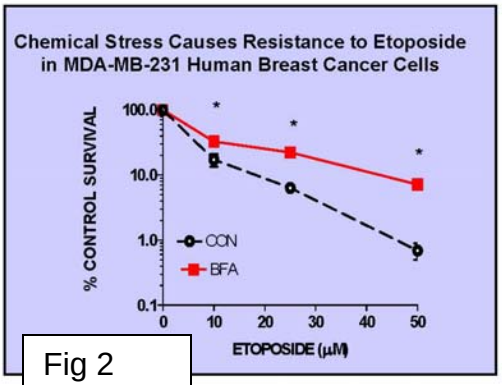

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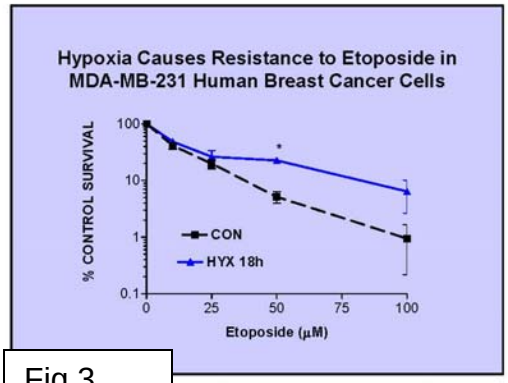

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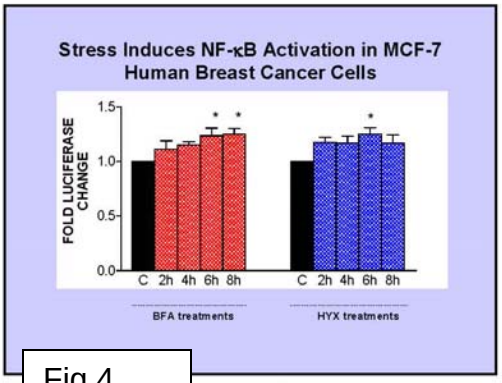

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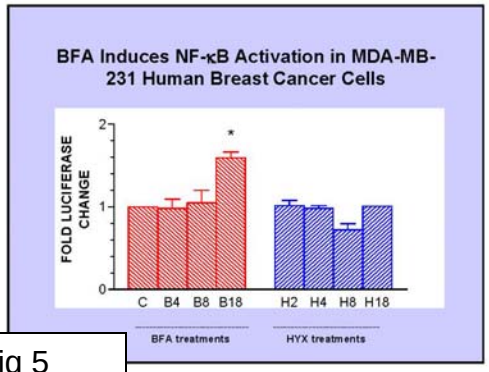

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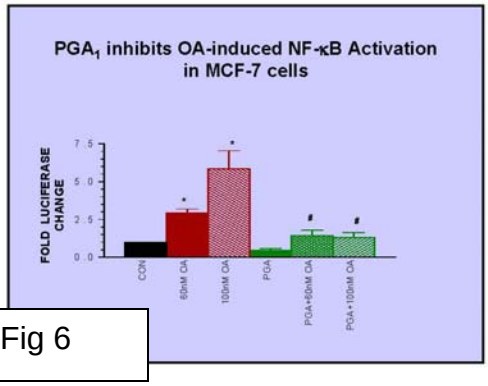

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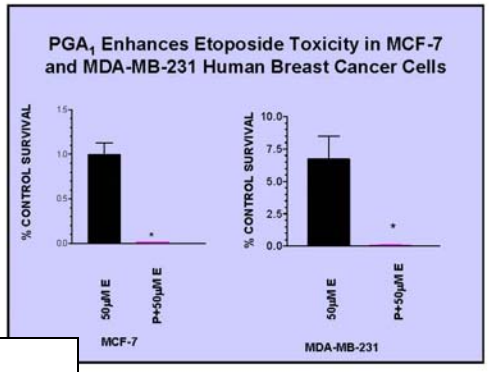

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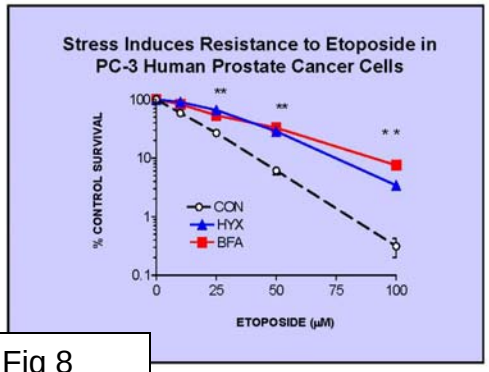

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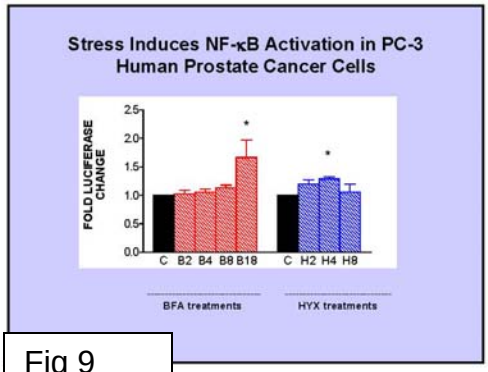

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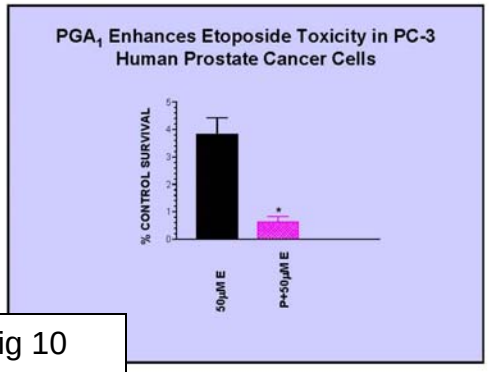
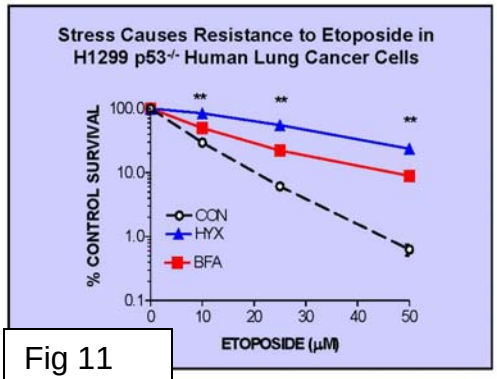


Fig 10

Both NF-kB and p53 pathways for cell survival contribute to cell decision making processes that determine apoptosis. Furthermore, p 53 can be activated by NF-kB. As shown by many, hypoxia also can activate p53 and that p53 activation is mediated through a different pathway than that used by DNA damaging agents. Thus, differences in cellular response to stress and NFkB activation could be due to differences in p 53 status. We assessed the role of p53 on stress induced resistance using a unique cell model, H1299 lung cells with an inducible p53 gene. Preliminary studies showed that the presence of the inducible p53 gene burden did not alter response to etoposide when p53 is off and that tetracycline, the inducing agent, did not alter responses to etoposide. Both physiologic and chemical induced stress induced NFkB and led to the development of resistance in H1299 (p53 -/-) lung cancer cells (Figures 11 and 12) Physiologic and chemical stress treated wt-p53 transfected H1299 cells develop resistance to etoposide when p53 is expressed (ON) or not expressed (OFF) (Figure 13). The presence of p53 (ON) shifts the dose response curve to the right but the response remains the same. PGA1 pretreatment, sensitizes H1299 cells to etoposide when p53 is ON or OFF. (Figure 14) These data suggest that p53 is not important for the stress induced resistance to topoisomerase II agents.



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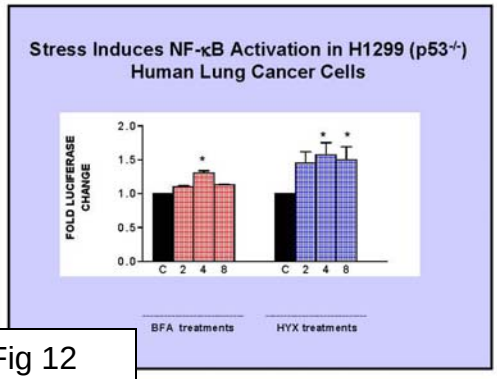

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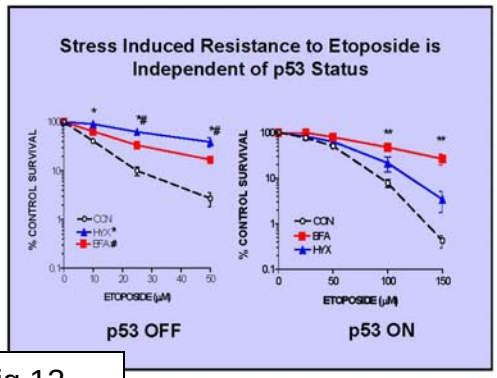

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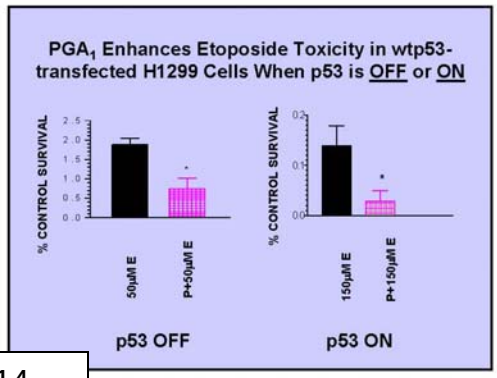
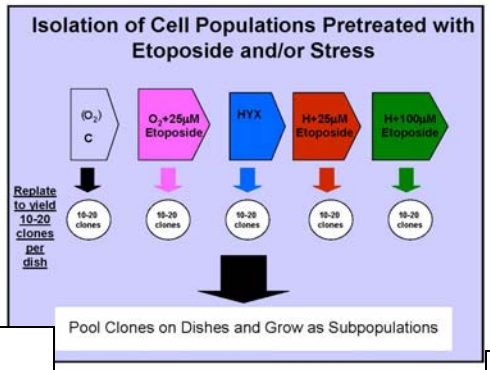


Fig 14

Hypoxia in solid tumors can be either chronic or transient. Transiently hypoxic regions arise because tumor blood vessels temporarily shut down by becoming compressed or obstructed by growth. Tumors therefore are repeatedly exposed to hypoxia and reoxygenation cycles. To determine if cells alter their responses to stress after a cycle of hypoxia and reoxygenation, we created tumor cell populations from cells treated with normal O_2, O_2 + 25 μM etoposide for 1h, hypoxia for 8 hours only (HYP), hypoxia for 8 hours + 25 μM etoposide for 1h (equimolar dose compared to O_2 group), and hypoxia for 8 hours + 100 μM etoposide for 1h (equitoxic dose compared to O_2 group). Cells were plated for survival such that 10-to 20 colonies per dish were expected and six dished per group were plated. After 14 days, colonies on the dishes were pooled together (100-200 colonies total per group), and then grown as a subpopulation. Each subpopulation was then either treated with hypoxia or BFA as a chemical stress (Figure 15) Survival to a test dose (50 μM) of etoposide was determined. Another cycle of stress, either chemical or physiologic stress, resulted in the development of a more resistant phenotype (Figure 16). A repeat of the entire

derivation of the subpopulations resulted in identical results (Figure 18). These results imply the hypoxia/reoxygenation cycles could result in enhanced stress resistance. This enhances resistance lasted several passages but gradually diminished with time such that by passage 8 (approximately 8 weeks later), cells resembled their non-stressed hypoxic counterparts.



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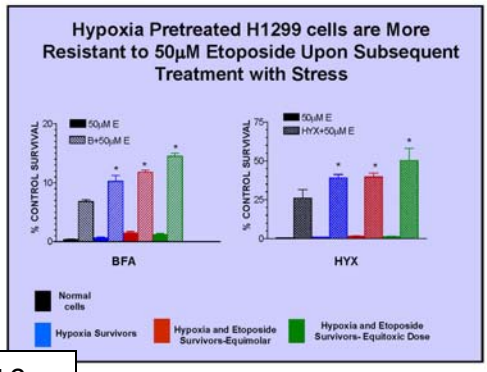

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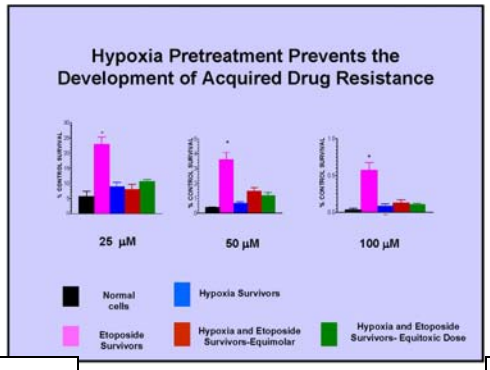


Fig 17

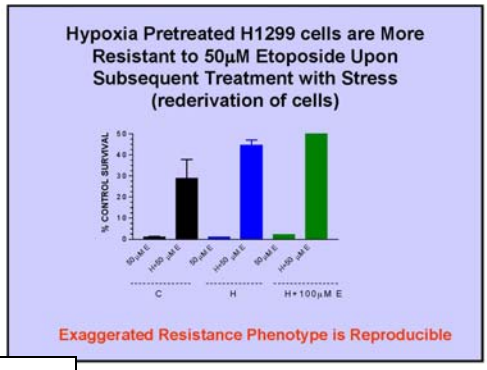


Fig 18

Subpopulations were also assessed using etoposide doses from 25-100 mM without any stress pretreatment. Subpopulation that had only seen control conditions continued to give normal expected responses to etoposide. The etoposide survivors (O_2 + etoposide) showed the development of etoposide acquired drug resistance. Hypoxia survivors in aerobic conditions were as sensitive to etoposide as the oxygenated normal cells. (as expected). More interestingly, the survivors from hypoxia + etoposide showed no acquired resistance, they showed survivals similar to normal cells (statistically not different). (Figure 17). These data suggest that hypoxia may be able to block the development of the acquired resistance to topoisomerase II agents.

The changes seen in the resistant pattern for the subpopulations were not due to changes cell growth patterns since the subpopulations exhibited similar growth curves (Figure 19). Further, a more robust NF-kB activation also did not appear to be the cause of the exaggerated resistant phenotype (Figure 20) since NFkB activation was similar in all subpopulations. Previously, we demonstrated that chemical stress and physiologic stress induced NFkB and also induced VEGF protein in breast cells (Figure 21 and 22). VEGF induction is primarily induced through the HIF-1 pathway. We therefore examined HIF-1 and VEGF in the subpopulations. HIF-1 was present in hypoxia exposed subpopulations without any additional stress and treatment with hypoxia caused HIF1 to translocate to the nucleus. (Figure 23 and 24). Treatment with hypoxia induced similar production of VEGF in control, hypoxia, and hypoxia + 100 μM etoposide survivors. These results suggest that exaggerated HIF1 responses are not responsible for the exaggerated phenotype.

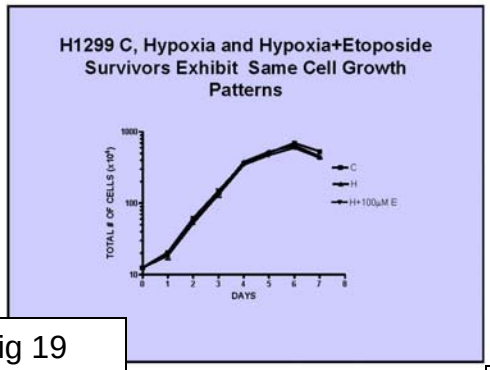
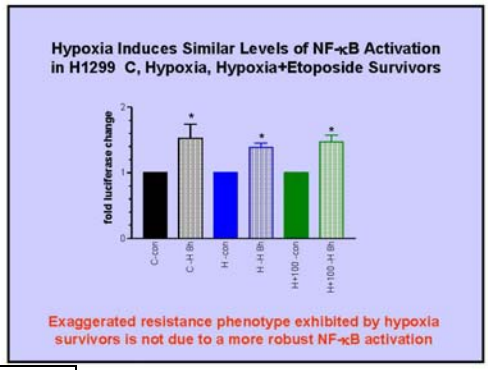


Fig 19



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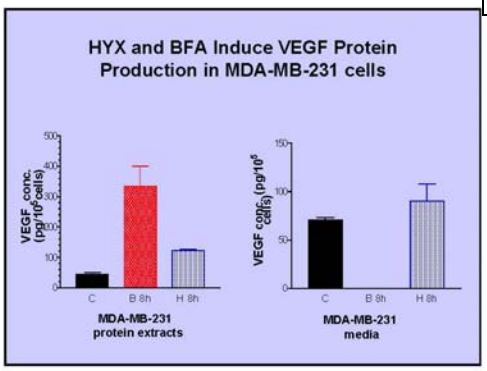
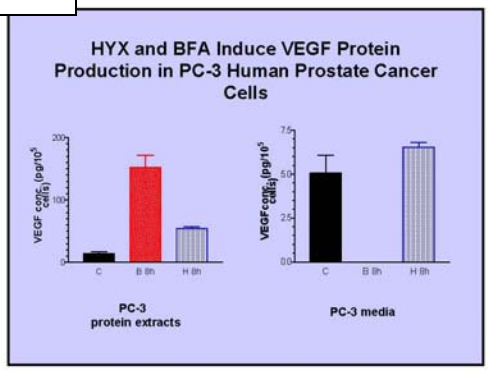


Fig 21



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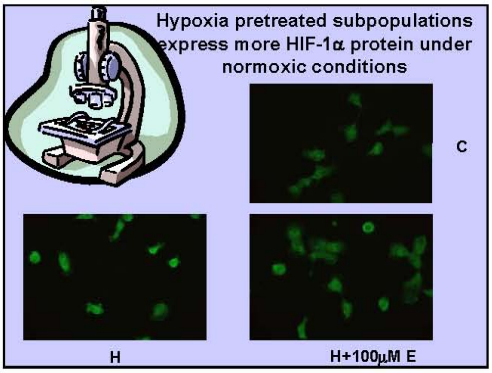


Fig 23

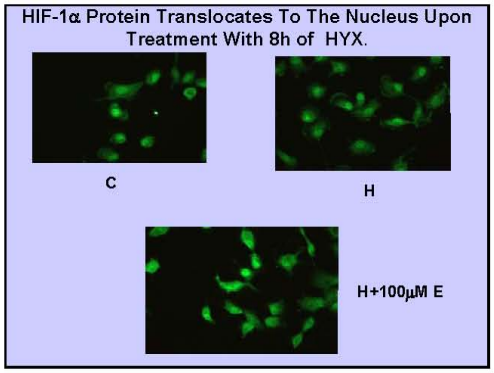
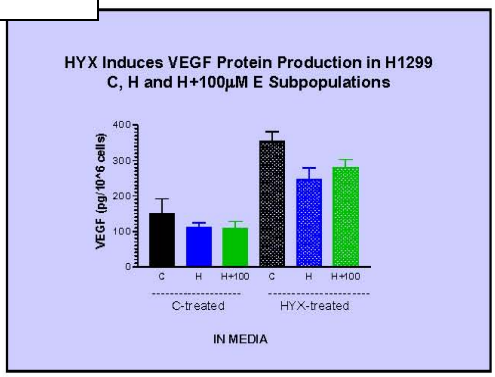


Fig 24



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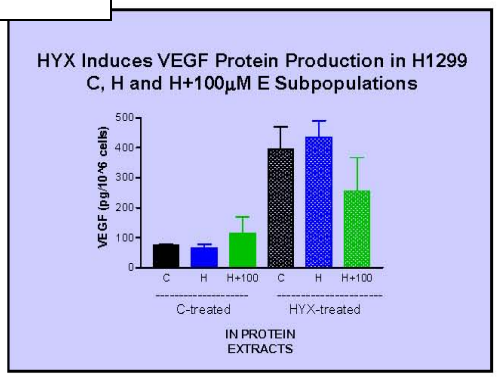

Fig 26

Additional work will be necessary to fully understand mechanisms for the exagerated resistance phenotype. However, these results have significant implications for therapeutic approaches for the treatment of solid tumors with topoisomerase II agents including the use of sensitizers like PGA1, the use of the proteosome inhibitor bortezomib, and the use of HIF-1 inhibitors as agents for improving therapeutic response.

KEY ACCOMPLISHMENTS

- Demonstrated that human breast tumor cell lines exhibit similar patterns of resistance to topoisomerase II inhibitors after physiologic or chemical induced stress.
- Demonstrated that human breast tumor cell lines respond to stress in a qualitatively similar fashion with activation of NFkB.
- Demonstrated that PGA1 could sensitize human breast cell lines to

topoisomerase II inhibitors.

- Demonstrated that human tumor cell lines such as PC3 and H1299 cells have patterns of response to stress similar to breast cell lines.
- Demonstrated that p53 status does not alter the response of cells to stress induced drug resistance but does shift the dose response curve.
- Demonstrate that p53 status does not alter sensitization to drug by NFkB inhibitors such as PGA1.
- Demonstrated that repeated exposure to stress (chemical or physiologic) results in an exaggerated more robust resistance to drug and that the response is more long lasting in that it does not disappear until after 8 passages.
- Demonstrated that hypoxia appears to blunt the development of drug induced acquired resistance.
- Demonstrated that repeated stress exposure cannot be explained by changes in NFkB activation, induction of HIF-1, or induction of VEGF since these are activated or induced to the same extent as control cells when stressed.

Reportable Outcomes

Presentations:

I S Hadjisavva, S R Patierno, and K A. Kennedy, NF-kB mediated stress-induced resistance to the topoisomerase II inhibitor etoposide in human cancer cells, American Association for Cancer Res 2003

LM Brandes, DA Stephan, K, Peterson, and KA Kennedy, Expression profiling of chemical and physiological stress treated breast tumor cells reveals a signaling pathway for drug resistance through NFkB activation, Era of Hope, US Army, 2002
I. S. Hadjisavva, J. Catania, M.L. Avantaggiati, and K.A. Kennedy, Stress induces Reistance to Etoposide in H1299 Human Lung Cancer Cells, American Association for Cancer Research Annual Meeting, Orlando FL, March 2004.

Conclusions

Our studies have demonstrated that physiologic stresses like hypoxia alters the responsiveness of mouse and human tumor cell lines to topoisomerase II inhibitors. Using inhibitors of the NFkB activation pathway, it is possible to sensitize cells to these agents. More importantly, our initial work with repeated stress exposure shows that the cellular sensitivity to topoisomerase II inhibitors is blunted even further. These studies have serious implications for treatment of cancer patients and suggest that use of NFkB pathway inhibitors to sensitize cells to drug should be beneficial. Indeed several groups during the course of these investigations demonstrated the usefulness of agents that affect the NFkB pathway in *in vivo* mouse tumor systems (Arit et al, 2001; Duffey et al, 1999; Cusack et al, 2000).

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Arit, A., Vorndamm, J., Bretenbroich, M., Folsch, U.R., Kalthoff, H., Schmidt, W.E., and Shafer, H. (2001) Inhibition of NFkB sensitizes human pancreatic carcinoma cells to apoptosis induced by etoposide or doxorubicin. Oncogene 20: 859-868.

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Introduction

The success of treatment for breast cancer is often limited by the development of drug resistance. We have described a unique type of resistance induced by the physiological factors at play within solid tumors. Solid tumors, due to inadequate vascularization contain microenvironmental regions that are hypoxic and thereby subject the neoplastic cells in these regions to cellular stress. The cellular response to this stress then determines cellular susceptibility to therapy. EMT6 mouse mammary tumor cells treated with hypoxia (paper in appendix) or the chemical stress agent brefedin A (BFA) induce resistance to agents that inhibit topoisomerase II (Lin et al, 1998, Brandes et al, 2001). Furthermore, data obtained through the present grant show that this chemically- and physiologic-induced resistance is mediated in large part by the nuclear transcription factor NF-κB. The overall scope of the work is to determine if NF-κB is the mediator of physiologic induced resistance and whether agents which alter NF-κB activation can alter the effectiveness of topoisomerase II type drugs.

Body

Tasks 1- 3 from **Year 1** were to insert the NF-κB p65 plasmid and then isolate, verify expression and determine drug resistance in a high and low expressor cell line. We have inserted both the p65 and p50 subunits into the ponasterone inducible vector system used previously to inhibit NfκB acivity in cells (for discussion of IκBαM results in the inducible vector system please see Brandes et al, Molecular Pharm. 2001, copy found in the appendix). Our previous data using this system has demonstrated that ponasterone A did not activate NF-κB in EMT6 cells, did not interfere with stress induced NF-κB activation, and was not toxic to the cells. Both vector p65 and p50 containing cells and empty vector cells were made for these experiments. Briefly, last year, we verified that ponasterone induced expression of p65 and p50 using western blot analysis for protein; showed that the induction of p65 and p50 in the cells led to enhanced NF-κB activation as measured by luciferase assays, and showed that p65 or p50 expressing cells were resistant to etoposide and doxorubicin, topoisomerase II inhibitors. When either p65 or p50 subunits were expressed, stress-induced activation of NF-κB was comparable to the unstressed, basal level of NF-κB activation and drug resistance was lower than in VCT (empty vector) controls. This abrogation of stress-induced activation was reflected in the lack of stress induced drug resistance during p65 or p50 expression. This type of result was surprising but has been seen in other stress pathways. For example, in the UPR pathway, mediated by unfolded protein, GRP78 (BiP) induction by stress is abrogated if in fact GRP78 is overexpressed (Dorner et al, 1987).

Additional investigation is required to determine the mechanism behind this finding. It may be that NF-κB activation by overexpressing either p65 or p50 may have simply

induced sufficient increases in IκB content in these cells such that stress could not induce NF-κB activation as efficiently. Indeed, our preliminary expression array data suggests that in fact IkB is induced during NF-κB activation as expected. We have not performed any array expression studies after p65 or p50 overexpression.

Task 4 and 5 from Year I concerned comparative experiments using an IκBαM which was not phosphorylatable and therefore could prevent the activation of NF-κB. The completed results from these studies are delineated in our paper which was published s in **Molecular Pharmacology**. A copy of the paper is in the Appendix. In brief, we showed that IκBaM expressing cells had similar sensitivities to etoposide as vector only expressing cells in the absence of stress; that stress would induced similar levels of drug resistance in both VCT and IκBaM cells (non-induced); that in IκBaM (induced)the cellular sensitivity to etoposide was similar to that of non-stressed cells and that biochemical measurements of NF-κB activation correlated with the cellular toxicity data (expression of IκBaM prevented stress-induced activation of NF-κB). This was true whether the stress used in the experiments was BFA (brefeldin A), HYP (hypoxia) or OA (okadaic acid). We conclude from the data from Task 1-5 that stress induced activation of NF-κB is the likely mechanism by which stress renders tumor cells resistant to drug induced toxicity.

In year 2, Tasks 1 and 2 were to compare drug resistance in transfected cell lines under conditions in which varying levels of expression were obtained. The original design was to isolate and use cloned with various levels of expression. To accomplish this with the inducible lines, we first attempted to determine if altering the concentration of inducing agent would alter the degree of resistance or the amount of resistance reversal that one would obtain. As seen below, our preliminary results with the IκBaM expressing cell line suggests that this is the case.

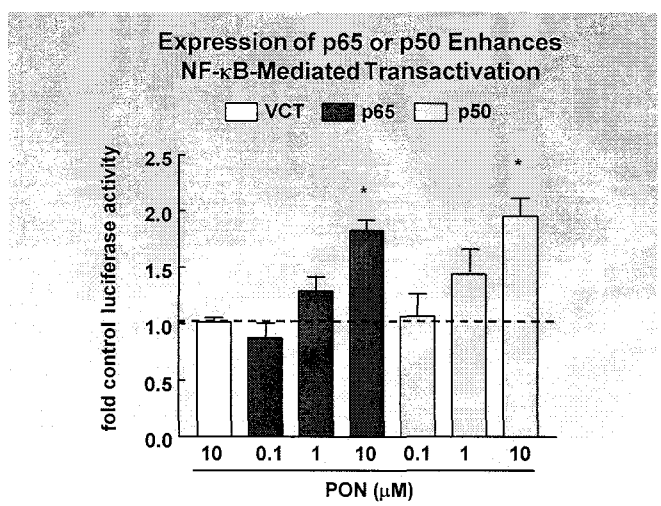
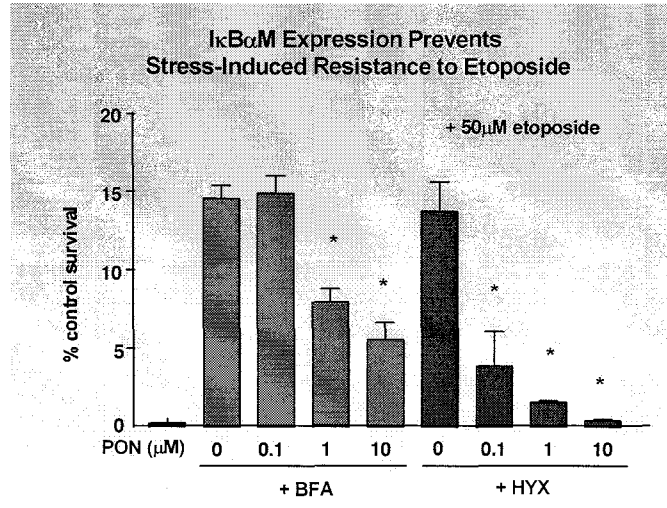


Figure A. Amount of Resistance Reversal is Dependant upon the Concentration of the Inducing Agent. IkBM cells were induced for 24 h with varying concentrations of PON and then treated with either BFA or hypoxia. Eight hours after stress treatment, cells were then treated for 1 h with

In addition,

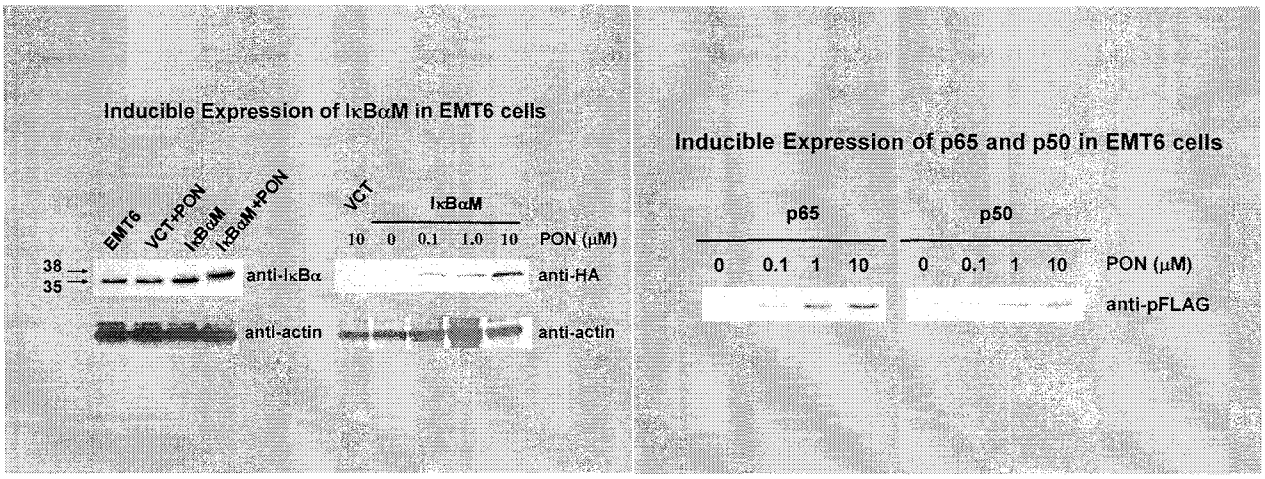
we have begun similar studies with p65 and p50 expressing cells lines as shown below in Figure B. The data also suggest that the level of resistance is dependent upon the concentration of ponasterone used for induction of the plasmid.

Figure B. Expression of p65 or p50 Induces Resistance to Etoposide. Cells were pretreated with varying concentrations of the plasmid inducing agent, Ponasterone (PON) for 24 h. The cells were then exposed to 50 μM etoposide for 1 h and cytotoxicity was assessed by colony formation. Cellular sensitivity to etoposide was dependent upon the amount of inducing agent used.



In addition, to show that induced activity is dependent upon expression, both western blot experiments and functional assays of NF-κB activity using the luciferase system were performed and showed that p65, p50 and IkBM were induced in a dose dependant manner.

Figure C: Expression of IkBMa or p65 or p50 is dependent upon the concentration of ponasterone used to induce the system. IkBMa has three HA tags and p65 or p50 has a FLAG tag. Cells were induced with various concentrations of ponasterone for 24 h and then collected for Western blot analysis.

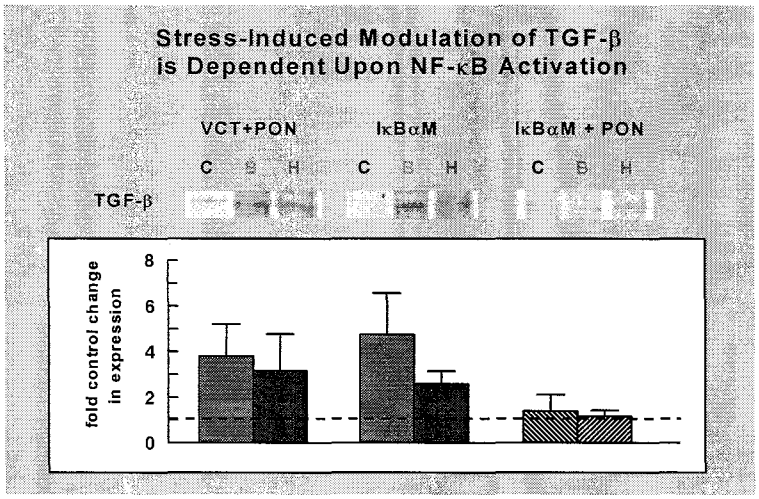


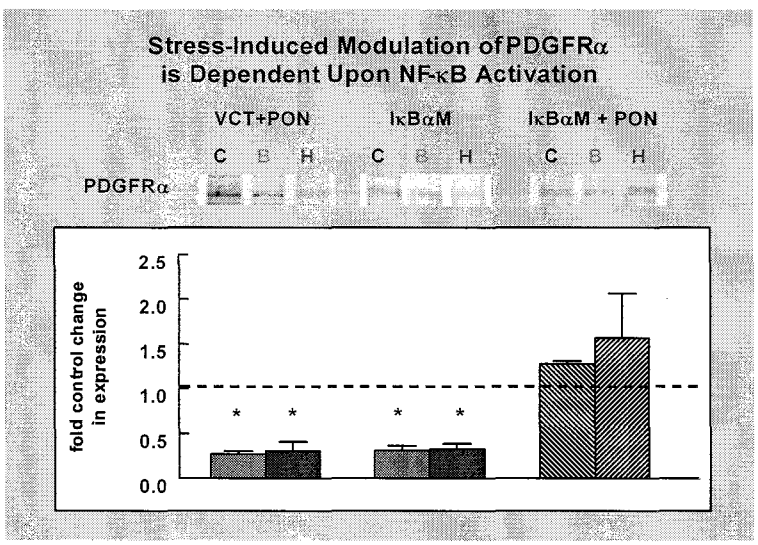
Our studies with the pharmacological inhibitor of NF-κB activation, prostaglandin A_1,(PGA_1) have been published in Oncology Research. These studies have demonstrated that stress treatment did not alter intracellular drug accumulation, topoisomerase II protein levels or inhibit topoisomerase II activity. Both hypoxia and BFA caused activation of NF-κB. Pretreatment of the cells with PGA_1 inhibited stress-induced activation of NF-κB and reversed BFA and hypoxia induced resistance. Resistance could be reversed when PGA_1 was given prior to or after the stress. These data imply that agents like PGA_1 which can abrogate the activation of NF-κB could be useful adjuncts to enhance the clinical efficacy of topoisomerase II directed chemotherapeutics. A copy of this manuscript is included in the appendix.

We have begun to determine the downstream signaling pathways that are involved in mediating resistant phenotype. The rationale behind these experiments is that understanding the downstream signals may allow us to refine the target. Because NF-κB is such a general transcription factor and plays a role in so many physiologic processes, the use of a general inhibitor may lead to a plethora of unintended effects and toxicities. Our hypothesis is that ER-stress via the ER overload pathway leads to the induction of a common subset of signals which mediate the resistance and that by comparing stresses and the reversal of stresses using expression arrays we may be able to focus in on a relatively small subset of genes. Our preliminary data which has not been fully analyzed suggests that this is the case. We have used the Affymetrix

mouse expression array system to perform initial assessments. Using matrix cross analyses of hypoxia and BFA with or without induction of IκBM induction, we can narrow the number of genes that represent the intersection of the two stresses to 40-60. In addition, initial classifications of the involved genes show that a wide variety of cell processes including cell cycle, energy metabolism, and apoptosis related genes may be involved. In addition, cursory inspection of the data show that expected gene products like IκB are increased during stress (NF-κB activation) and decreased during expression of IκBM during stress (inhibition of NF-κB activation). These data are to be presented, in a poster presentation at the ERA of Hope Meeting in Orlando FL "Exparession profiling ochemical and physiological stress treated breast tumor cells reveals a signalig pathway for drug resistance through NfkB activation" and "Expression profiling reveals a role for TGF-beta and the PDGFRa/MAPK signaling pathway in the development of stress-induced drug resistance". Copies of these posters can be found in the Appendix.

These studies have demonstrated that expression profiling is a useful methodology to assess complex changes in gene expression provided that additional methods are used to verify and corroborate the studies. For example, stress via hypoxia and brefeldin A elevated the expression of IkB as expected, VEGF, and NO synthetase. In addition we found that TGF-beta was elevated, PDGFRa and MEK 1 / 2 were decreased in expression profiles. Western blotting demonstrated that TGF-beta protein was increased, PDGFRa protein was decreased but that MEK1 and MEK2 were unaffected. However, the phosphorylated form of MEK was decreased. Additional studies with inhibitors showed that inhibiting TGF-beta, blocking the PDGFRa receptor or inhibiting the function of phosphorylated MEK all resulted in drug resistance to etoposide. To determine if these genes might be involved in NFkB dependent resistance, we assessed the status of these proteins when IkBM was induced in stressed cells. Induction of IkBM blocked the stress dependant changes in TGF-beta expression, PDGFRa, and phosphoMEK (see figure D)





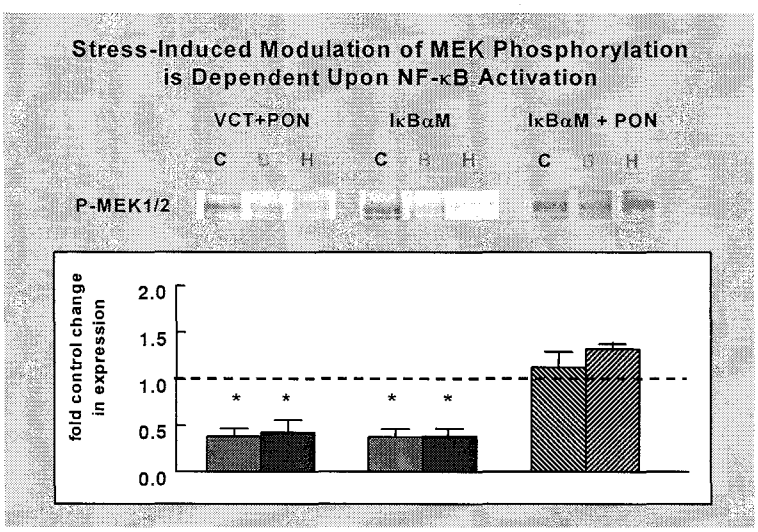


Figure D. Western blot analysis of TGF-beta, PDGFRa and phosphorylated MEK 1/ 2 in stressed EMT6 cells with or without I κBM induction with ponasterone A. Protein levels from stressed- induced empty vector cells (VCT) are shown for comparison.

Task 6 from year 1 and Tasks 3 and 4 from year 2 concern the effects of NF-κB inhibitors on *in vivo* tumors. Again this year, these studies have not begun. Although it was initially thought that the contamination of the mice from mouse hepatitis virus would be easily contained and eliminated, this has proven to be more difficult. The institution concluded that in order to maintain clean animals it would need to build a barrier facility. The facility is now complete and can be utilized. We have requested and received a one year extension of this project to allow us to complete this work.

Key Research Accomplishments

- Demonstrated that the dose dependent induction of the p65, p50 and IkBM transfected cells by ponasterone resulted in the dose dependent expression of the proteins and effects on drug resistance to etoposide.
- Showed that dose dependent induction of p65 or p50 cells by ponasterone led to increased NF-κB activity as measured by elevation of luciferase.
- Used expression arrays to begin to dissect the common downstream pathways for NF-κB mediated drug resistance.
- Demonstrated that stress induced activation of NF-κB controls TGF-beta expression in these cells and leads to a signal transduction pathway that contributes to drug resistance.
- Have identified 40-80 genes that play a possible role in mediating resistance and resistance reversal.

Reportable Outcomes

Presentations:

L.M. Brandes, S. R. Patierno, D.A. Stephan, and K.A. Kennedy, Expression analysis reveals a role for TGF-beta and the PDGFRa/MAPK signaling pathway in the development of both chemical and physiologicial induced drug resistace of breast cancer cells, Proc. Amer Assoc. Cancer Research 53: 5371, 2002

KA Kennedy, LM Brandes, and DA Stephan, Expression profiling of chemical and

physiologic stress-treated breast tumor cells reveals a signaling pathway for drug resistance through NF-κB activation, 41st Annual Meeting of the Amer. Soc. for Cell Biology, Abst. 108, 2001.

Papers:

L. M Brandes, Z.P. Lin, S. R. Patierno, and K.A. Kennedy, Reversal of physiological stress-induced resistance to topoisomerase II inhibitors using an inducible phosphorylation site-deficient mutant of IκBα, Molecular Pharm. 60: 1-9, 2001.

Y.C. Boller, L.M. Brandes, R. L. Russell, Z. P. Lin, S. R. Patierno, and K. A. Kennedy, Prostaglandin A_1 inhibits stress-induced NF-κB activation and reverses resistance to topoisomerase II inhibitors, Oncology Res. 12: 383-395, 2001.

Cell Lines:

Stable ecdysone inducible expression of p65 in the EMT6 mouse mammary tumor cell line- EMT6-p65.

Stable ecdysone inducible expression of p50 in the EMT6 mouse mammary tumor cell line- EMT6-p50.

CONCLUSIONS

Our results show that NF-κB activation by physiologic stress leads to resistance to topoisomerase II type drugs including etoposide, teniposide, and doxorubicin. Pharmacologic agents which interfere with NF-κB activation can reverse resistance when given before, during or after stress. Genetic manipulation of the IκBα subunit to block NF-κB activation render the cells completely sensitive to drug during stress. Overexpression of either the p50 or p65 subunits of the transcription factor also leads to drug resistance. Furthermore, expression analysis reveals that there is an NF-κB dependent pathway that directly influences TGF-beta expression that ultimately results in a decrease in MEK phosphorylation. These results confirmed by western blots suggest additional inhibitors that interfere with this pathway could alter the drug resistant phenotype. Indeed, pharmacologic manipulation to mimic these changes demonstrated that drug resistance was induced; the resistance that was induced was small suggesting that additional pathways downstream of NF-κB are involved in the development of the resistant phenotype. Importantly, these data show that NF-κB expression is sufficient and necessary to cause cells to become resistant to topoisomerase II inhibitors. Manipulation of tumor NF-κB activation or manipulation of the downstream signaling events arising from NF-κB activation should lead to altered

responsiveness to topoisomerase II inhibitors.

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Year 1

Introduction

The success of breast cancer treatment is often limited by the development of drug resistance. A unique form of drug resistance has been described that is induced by the physiological conditions associated with solid tumors. EMT6 mouse mammary tumor cells treated with hypoxia or the chemical stress agent brefeldin A (BFA) induce resistance to agents that inhibit topoisomerase II (Lin *et al*., 1998). Our data have shown that hypoxia- and BFA-induced resistance to topoisomerase II inhibitors is not mediated by alterations in p-glycoprotein, drug concentration, or topoisomerase II activity. Our recent data suggest that chemically-induced resistance is mediated by the nuclear transcription factor NF-_B (Lin *et al*., 1998). The overall scope of the work is to determine if NF-κB is the mediator of physiologic induced resistance and whether agents which alter NF-κB activation can alter the effectiveness of topoisomerase II type drugs.

Body

Six tasks were proposed for this first year of the award. **Tasks 1, 2 and 3** were to insert the NFκBp65 plasmid and then isolate, verify expression and determine drug resistance in a high and low expressor cell line. While we were able to obtain cell lines transfected with the NF-κB plasmid for p65 or the alternative subunit, p50, the actual expression of p65 or p50 was not elevated and in fact appeared to be lower. Upon treatment with stress, the activation of NF-κB was actually lower not higher as measured by the κB dependant luciferase reporter gene. This anomalous result was puzzling. However, at the AACR meetings, another group reported a similar result in that overexpression of p65 resulted in down regulation of a NF-κB dependant gene. To circumvent this phenomenon, we will insert the p65 and p50 subunits into the ecdysone inducible system and then create EMT6 cell lines in which p65 and p50 are induced by ponasterone A. As will be seen below, we have successfully used this system to create an EMT6 cell line in which the dominant negative mutant of IκB is induced by ponsterone treatment.

Task 4 was to transfect into EMT6 cells the non-phosphorylatable mutant of IκB to produce a dominant negative mutant EMT6 cell line. **Task 5** was to evaluate the responses of the cell lines to stress. We were successful again in putting the IκBαM sequence under a hygromycin selectable CMV promoter and transfecting it into cells. However, the clones that were selected did not contain the IκBαM gene. We carried out the transfection protocols 3 times; and were unsuccessful all three times. In fact, close observation of the transfected cells as well as experiments with transient tranfection studies suggested that EMT6 cells were highly dependent upon the NFκB response in order to survive the stress of transfection and selection. Therefore, we reasoned that a more profitable route to pursue was that of using an inducible vector. Many of the inducible vectors that are available use inducing agents that actually activate NFκB.

Therefore, we did initial experiments to demonstrate that the ecdysone inducible system did not alter the activation of NFκB. These experiments showed that the inducing ligand, ponasterone A, did not activate NFκB as measured by changes in gel shift assays and κB dependent luciferase reporter assays. Furthermore, we also determined that ponasterone A did not interfere with stress induction of NFκB and that ponasterone A was also not toxic to EMT6 cells as measured by colony forming cytotoxicity assays.

We then constructed the pIND-IκBαM plasmid and co-transfected the pIND-IκBαM plasmid with the pVgRXR (containing the ecdysone receptor) at a ratio of 1:5 into EMT6 mouse mammary tumor cells. Empty vector cells were also made and used as controls. Cells were selected with hygromycin and several colonies were obtained and isolated. Briefly, we verified that ponasterone induced expression of IκBαM in the cells; that expression of the IκBαM protein prevented NF-κB activation by brefeldin A (BFA), hypoxia (H), or okadaic acid(OA); that the presence of the IκBαM vector or the expression of IκBαM protein in the cells did not alter the cytotoxic effects of etoposide in EMT6 cells in the *absence* of stress; that hypoxia and brefeldin A both induced resistance in EMT6 cells containing either the vector only construct or the uninduced IκBαM construct; and that induction of IκBαM expression with ponasterone A prevented the development of stress-induced resistance (induced by hypoxia or brefeldin A) to etoposide. In fact, ponasterone A induced cells were not statistically different from non-transfected EMT6 cells in their susceptibility to etoposide. We are currently investigating the cytotoxic effects of doxorubicin in our unique cell lines. Detailed results of these experiments are found in the reprint of the poster presented on this work at the 91st Annual meeting of the American Association for Cancer Research and can be found in the Appendix. These data give further support to our hypothesis that NFκB mediates stress induced drug resistance and show the power of using the inducible expression system for studying stress induced drug resistance. It should be noted that with these data we have mostly completed Task 2 from year 2 as well.

Furthermore, we have continued to confirm and enhance the preliminary data we had with prostaglandin A_1 and have demonstrated that this compound which reverses drug resistance does interfere with NFκB activation in EMT 6 cells. In addition, the data show that prostaglandin A_1 can reverse chemical or physiological induced resistance to teniposide and etoposide; can prevent or partially prevent the stress resistant phenotype even if given prior to or after the stress; does not interfere with the activity of topoisomerase II, and that stress does not interfere with or alter the uptake of etoposide by the cells. These results were also reported at the 91st Annual meeting of the American Association for Cancer Research. Detailed results can be found in the reprint of the poster presentation in the Appendix. They further strengthen our understanding of the effects of prostaglandin A_1 in our system and prepare us better to determine *in vivo* activity of prostaglandin A_1 as a reversal agent.

Both of these presentations are being prepared for submission for publication.

Task 6 was to determine the maximal dose for Balb c mice and to begin *in vivo* studies. We have not begun these studies. The animal quarters has been plagued by mouse

hepatitis virus and as a consequence, we have not had animals shipped in. This issue is resolving and other investigators with mice in the facility have found that the mice are no longer testing positive for elaboration of virus. Further sero testing is necessary to ensure that the subsequent mice are negative. We will probably be able to commence these studies in the Fall of 2000. We anticipate that these studies should be successful. In support of our hypothesis and the appropriateness of pursuing our *in vivo* studies, Ogiso et al. (Cancer Res. 60: 2429-2434, 2000) showed that the use of lactacystin, a proteosome inhibitor, prevented stress induced etoposide resistance *in vivo* and *in vitro*. These data *in vitro* are similar to our previously published work with the proteosome inhibitor, MG132.

Recommended Changes to the Statement of Work for Year 2:

1. Use the inducible expression system to express p65 and p50 in EMT6 cells and then compare cytotoxicity responses to etoposide in vector and ponasterone induced cells.

2. Perform the tumor sensitivity studies with prostaglandin A_1.

Key Research Accomplishments

- Determined that prostaglandin A_1 inhibited both brefeldin A and hypoxia induced activation of NFκB
- Determined the prostaglandin A_1 reversed both hypoxia and brefeldin A induced toxicity to etoposide and teniposide. Prostaglandin A_2 can cause partial or complete reversal when given prior to or up to 7 hours after stress.
- Prostaglandin A_1 does not alter topoisomerase II activity directly.
- The stress agent, brefeldin A does not directly effect topoisomerase II activity directly nor does it alter drug uptake in cells.
- Constructed the pIND-IκBαM plasmid which contained the nonphosphorylatable form of IκB.
- Transfected pIND-IκBαM and pVgRXR plasmids into EMT6 cells and isolated hygromycin resistant clones.
- Showed that ponasterone A, the ecdysome receptor ligand, does not alter NFκB activation and does not effect long term survival of EMT6 cells.
- Showed that ponasterone A induction of IκBαM cells resulted in the expression of IκBαM protein and that ponasterone A induction of IκBαM cells resulted in the inhibition of stress-induced activation of NF-κB.

- Showed that induction of IκBαM cells resulted the diminution of measurable NFκB complexes in gel shift assays with brefeldin A, hypoxia or okadaic acid as the stress agent.

- Induction of IκBαM in nonstressed cells did not alter cellular sensitivity ot etoposide per se.

- Induction of IκBαM in stressed cells reversed stress induced resistance to teniposide.

REPORTABLE OUTCOMES

Presentations:

L. M. Brandes, Y. C. Boller, Z-P. Lin, S. R. Patierno, and K.A. Kennedy, NF-κB mediates stress induced resistance to etoposide. Proc. Amer. Assoc. for Cancer Res. 41: 751, 2000.

Y. C. Boller, R. L. Russell, L. M. Brandes, Z-P. Lin, S . R. Patierno, and K. A. Kennedy, Prostaglandin A_1 reverses stress-induced resistance to teniposide, Proc. Amer. Assoc. Cancer Res. 41: 867, 2000.

Cell Lines:

Stable ecdysone inducible expression of IκBαM in EMT6 mouse mammary tumor cell line –EMT6-IκBM.

Funding applied for based upon work supported by this award:

Lori M. Brandes, Predoctoral Award, US Army BCRP, " Altherations in gene transcription by pphysiological stress: a mechanism for drug resistance through NFκB actuvation", Submitted June, 2000.

Reserach opportunities applied for and/or received on experiences/training supported by this award:

Lori M. Brandes, Fellowship Award from the Washington Metropolitan Chapter of the Achievement Rewards for College Scientists (ARCS), 2000-2001.

CONCLUSIONS

Our results so far show strong and convincing pharamcologic and genetic evidence that NFκB mediates physiologic induced drug resistance. Because cancer cells in solid breast tumors exist in abnormal, physiological-based stress environments, our results imply that one can take these intrinsically resistant cells and sensitize them to topoisomerase II directed drugs like etoposide by interfering with the activation of NFκB. Our studies demonstrate that one can even sensitize cells that have already been exposed to stressful environments. These data suggest improved therapies for patients if a topoisomerase II dependent agent is combined with an anti- NFκB type agent.